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Mouse models are very valuable for preclinical testing of new therapeutic drugs against breast cancer and for studying how breast cancer cells invade and spread, but most mammary tumors arising in mice are different from human breast cancer. Most mouse models are created by targeting oncogenic lesions primarily to the more mature differentiated breast cells. Clinical breast cancer, however, are believed to arise from undifferentiated progenitor cells. Therefore, if we can induce mouse breast tumors specifically from progenitor cells, we may be able to produce models that more closely resemble human breast cancer. No promoter that is exclusively expressed in the mammary progenitor cells has yet been reported, but the promoter of keratin 6 is a likely candidate.

In order to determine whether keratin 6 is indeed a progenitor cell marker, and whether targeting oncogenes to progenitor cells may yield better models, we have succeeded in creating a BAC transgenic line expressing from the keratin 6 promoter the TVA viral receptor, for selective targeting of reporters and oncogenes using a viral vector RCAS. Next, we will use this line of mice to determine whether these K6-positive cells are indeed progenitor cells, and whether they will give rise to tumors that better resemble human breast cancers.

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Developing a novel mouse model for breast cancer by targeting oncogenes to mammary progenitor cells

Yi Li, Ph.D.

INTRODUCTION.

Mouse models are very valuable for preclinical testing of new therapeutic drugs against breast cancer and for studying how breast cancer cells invade and spread, but most mammary tumors arising in mice are different from human breast cancer. Unlike their human counterparts, mouse mammary tumors from current mouse models usually lack response to anti-hormone therapies, fail to metastasize to the bone, and rarely display chromosomal abnormalities, limiting the use of current models for animal testing of novel therapeutic drugs, and restrict the value of these models for dissecting the molecular pathways leading to aneuploidy, hormone resistance, and cancer metastasis to the bone.

Most mouse models are created by targeting oncogenic lesions primarily to the more mature differentiated breast cells. Clinical breast cancer, however, are believed to arise from undifferentiated progenitor cells. Therefore, if we can induce mouse breast tumors specifically from progenitor cells, we may be able to produce models that more closely resemble human breast cancer. No promoter that is exclusively expressed in the mammary progenitor cells has yet been reported, but the promoter of keratin 6 is a likely candidate based on our recent finding and the unpublished work of Dr. Jeff Rosen.

We hypothesize that keratin 6 is a marker of breast progenitor cells and that targeting oncogenes using this promoter may produce breast cancer models that more closely resemble human breast cancer. We also plan to determine if tumors arising from keratin 6-positive mammary cells will differ in phenotype depending on the initiating oncogenes.

BODY

Task 1. **To demonstrate that keratin 6 is a marker for mammary progenitor cells. Months 1-12**

a. Stain 3 sections of mammary glands from 3 pubertal females (3 week-old) for co-expression of keratin 6a and ER, PR, keratin 8, keratin 14, Sca-1, and peanut lectin to define the differentiation status of keratin 6-positive cells in mammary glands. Months 1-6. We have developed assays for immunostaining for these markers. We have found that K6 is occasionally colocalized with K8, but never α-SMA.
b. Create transgenic mice expressing GFP driven by the keratin 6 promoter. Screen for founder line that express GFP only in keratin 6-positive cells. Months 1-9. We have created founder lines for BAC transgenic expression of GFP from keratin 6 promoter. The founder lines are being screened for expression of GFP in mammary cells that express keratin 6.
c. Transplant various amount of isolated GFP-positive mammary cells into cleared fat-pads to ascertain that GFP-positive mammary cells can regenerate ductal tree. Months 9-12. To be done.

Task 2. **To target breast cancer genes to mammary progenitor cells. Months 1-30**

a. Create transgenic mice (in a FVB/N strain) expressing the reverse tetracycline-regulated transactivator (rtTA) using the keratin 6 promoter. Breed at least five founder lines with established tetO-β-galactosidase mice to identify one that induces β-galactosidase only in keratin 6-positive cells. Months 1-12. We have obtained and rederived into our facility this line of mice from another investigator Tom O'Brien (1). We are in the process of testing this line for coexpression of rtTA and K6. If they are not co-expressed, we will make BAC transgenic mice so that rtTA will be selectively expressed in K6+ cells.

b. Breed K6-rtTA transgenic mice with established tetO-c-Myc mice. Transplant the mammary cells from the resulting bi-transgenic mice into 10 inguinal mammary fat-pads in 10 FVB/N mice. Induce c-Myc expression in the mammary gland by Dox. Monitor mice for mammary tumors in the transplanted fat-pads. Months 12-24. To be done.

c. Determine the histopathology, metastatic spread, aneuploidy, levels of ER/PR, and cell differentiation status for the resulting tumors. Compare these features with those of mammary tumors from MMTV-c-Myc to determine if the tumors arising from keratin 6-positive cells will more closely resemble human breast cancer. Months 24-30. To be done.

d. Create transgenic mice expressing TVA driven by the keratin 6 promoter. Screen founder lines for one that expresses TVA only in keratin 6-positive cells. Breed K6-TVA transgenic mice with the already established p53 floxed mice to produce females that are K6-TVA /p53flox/flox for the experiments below. Months 1-12. We have successfully created BAC transgenic mice expressing TVA from the keratin 6 promoter (Figure 1). The TVA expression appears to be limited to keratin 6-positive cells. We are doing further work to prove that. The mice are being bred with p53 floxed mice.

e. Isolate mammary cells from these mice. Infect them with our established RCAS-K-RasG12D-IRES-Cre to express K-RasG12D and to delete p53. Transplant the infected cells to 10 mammary fat-pads in 10 wild-type mice. Monitor the recipient mice for development of mammary tumors. Months 12-24. To be done.

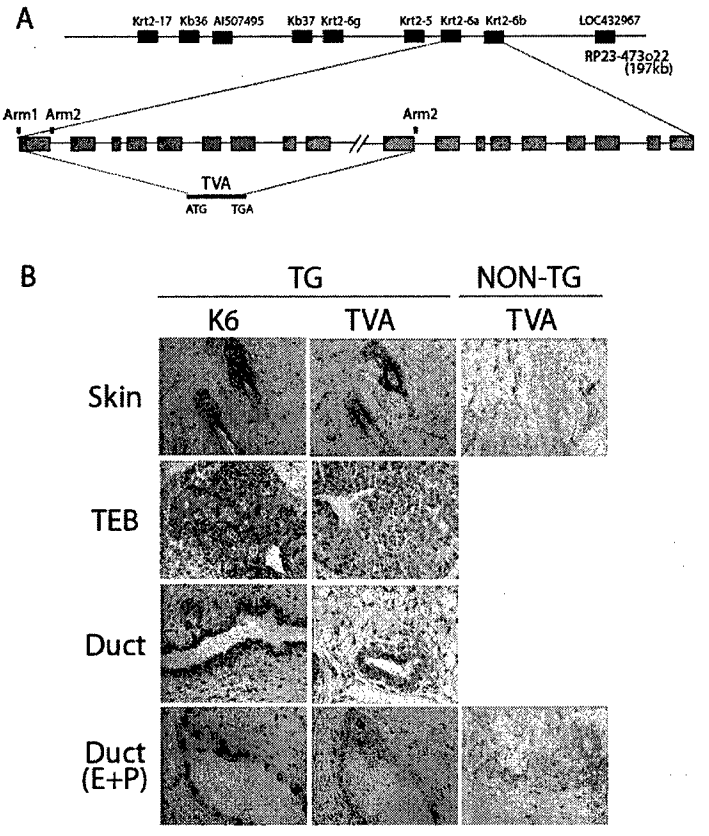

Figure 1. Creation of K6a-TVA BAC transgenic mice. (A) The BAC transgenic construct. (B). The expression of TVA appears to be limited to cells stained positive for K6. Since the K6 antibody stains both K6a and K6b. It is not surprising that we see more K6-positive cells than TVA-positive cells.

f. Inject concentrated RCAS-K-RasG12D-IRES-Cre virus to inguinal mammary glands of 10 pubertal and 10 estrogen-stimulated K6-TVA /p53flox/flox bi-genic females. Monitor the injected mice for development of mammary tumors. Months 12-24. We have succeeded in using this line of mice for introduction of oncogenes. As a proof of principle, we have demonstrated that we can use RCAS viruses expressing PyMT to induced mammary tumors, which are different than MMTV-PyMT-induced tumors, suggesting that progenitor cell derived tumors may be different than tumors induced from other cell types. Next we will test RCAS viruses expressing other oncogenes and Cre to deleted floxed p53. Since we can induce tumors from in vivo infection, it is no longer necessary to do ex vivo infection and transplantation.

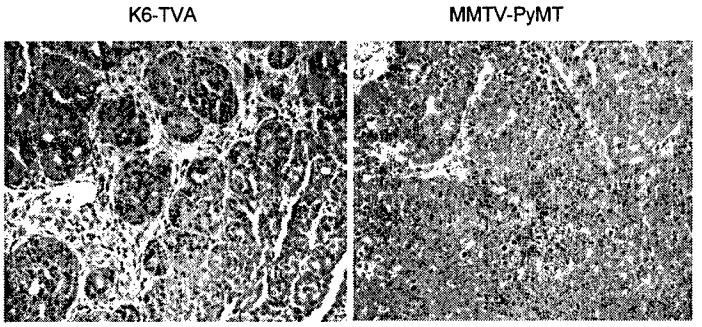

Figure 2. Mammary tumors induced in pubertal K6-TVA transgenic mice using RCAS-PyMT viruses are different than tumors arising in MMTV-PyMT transgenic mice. Please note that tumors at the left are more acinar, suggesting progenitor cell-derived tumors may retain higher potential to differentiate.

g. Inject concentrated RCAS-K-RasG12D-IRES-Cre virus into 20 inguinal nipple ducts or segmental ducts of 10 K6-TVA /p53flox/flox bi-genic females. Monitor injected mice for formation of mammary tumors. Months 12-24. To be done.

h. Determine the histopathology, metastatic spread, aneuploidy, levels of ER/PR, and cell differentiation status for the resulting tumors in 2.e through 2g. Months 24-30. To be done.

An unexpected finding:

The above proposed approaches were set up to test whether cancers arising from K6-positive cells more closely resemble human breast cancer. During the past one year, we were surprised to find that the MMTV-Wnt-1 transgenic model, which we had previously reported to develop cancers from progenitor cells, mostly give rise to ER-positive mammary cancers. Since most other transgenic models, which target oncogenes to more differentiated cells, develop ER^- tumors, this finding suggests that oncogenes targeted to progenitor cell may indeed yield valuable models. Further tests establish this model as the first model of ER^+ breast cancers that are de novo resistant to antiestrogens. In addition, we have found that secondary mutations can modulate ER expression in this model, providing a mechanistic explanation why there are both types of breast cancers. A more detailed description of this finding is described in our paper (2), which is appended.

Task 3. **To determine if the phenotypic properties of mammary tumors will be dictated by the initiating oncogenes targeted to progenitor cells. Months 12-36.**

a. Breed K6-rtTA transgenic mice with established tetO-K-RasG12D mice. Transplant the mammary cells from the resulting bi-transgenic mice into the mammary glands of 10 FVB/N mice. Induce K-Ras expression in the mammary gland by Dox. Monitor mice for mammary

tumors in the transplanted fat-pads. Months 12-24. To be done.

b. Determine the histopathology, metastatic spread, aneuploidy, levels of ER/PR, and cell differentiation status for the resulting tumors. Compare these features with those of mammary tumors from inducible expression of c-Myc (see 2b and 2c) to determine if these two initiating oncogenes will lead to mammary tumors of different phenotype. Months 24-36. To be done.

c. Generate mammary tumors by infecting mammary cells of K6-TVA/p53flox/flox mice with RCAS-c-Myc-IRES-Cre. Use the most successful method of infection as determined in 2e through 2g for infection of 10 experimental mice. Months 12-24. To be done.

d. Determine the histopathology, metastatic spread, aneuploidy, levels of ER/PR, and cell differentiation status for the resulting tumors. Compare these features with those of mammary tumors from inducible expression of c-Myc (see 2h) to determine if these two initiating oncogenes will lead to mammary tumors of different phenotype. Months 24-36. To be done.

KEY RESEARCH ACCOMPLISHMENTS:

- Published one original research paper and one review paper.
- Created BAC transgenic mice expressing TVA from the keratin 6 promoter.
- Demonstrated that introduction of oncogenes into keratin 6-positive cells can induce mammary tumors.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

1. One original paper. (Zhang X, Podsypanina K, Huang S, Mohsin SK, Chamness GC, Hatsell S, Cowin P, Schiff R, Li Y. Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. Oncogene. Epub. 4/11, 2005.)
2. One review article. (Li, Y., and Rosen, J.M., Stem/progenitor cells in mouse mammary gland development and breast cancer. *J. Mammary Gland Biol & Neoplasia*, In press.)
3. One invited talk at AACR on 4/19/2005. Title: Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations.
4. Created BAC transgenic mice expressing TVA from the keratin 6 promoter.

CONCLUSIONS: We have created critical reagents and mouse models for this project and have succeeded in inducing tumors from one of the transgenic lines. In addition, we have discovered a mouse model of antiestrogen resistant ER^+ breast cancers.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

1. Guo Y, Zhao J, Sawicki J, Peralta Soler A, O'Brien TG. Conversion of C57Bl/6 mice from a tumor promotion-resistant to a -sensitive phenotype by enhanced ornithine decarboxylase expression. Mol Carcinog 1999;26(1):32-6.

2. Zhang X, Podsypanina K, Huang S, Mohsin SK, Chamness GC, Hatsell S, et al. Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. Oncogene 2005.

APPENDICES:

Zhang X, Podsypanina K, Huang S, Mohsin SK, Chamness GC, Hatsell S, Cowin P, Schiff R, Li Y. Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations. Oncogene. Epub. 4/11, 2005.

Li, Y., and Rosen, J.M., Stem/progenitor cells in mouse mammary gland development and breast cancer. *J. Mammary Gland Biol & Neoplasia*, In press.

Oncogene (2005), 1–12

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ORIGINAL PAPER

Estrogen receptor positivity in mammary tumors of *Wnt-1* transgenic mice is influenced by collaborating oncogenic mutations

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The majority (75%) of human breast cancers express estrogen receptor (ER). Although ER-positive tumors usually respond to antiestrogen therapies, 30% of them do not. It is not known what controls the ER status of breast cancers or their responsiveness to antihormone interventions. In this report, we document that transgenic (TG) expression of *Wnt-1* in mice induces ER-positive tumors. Loss of *Pten* or gain of *Ras* mutations during the evolution of tumors in *Wnt-1* TG mice has no effect on the expression of ER, but overexpression of *Neu* or loss of *p53* leads to ER-negative tumors. Thus, our results provide compelling evidence that expression of ER in breast cancer may be influenced by specific genetic changes that promote cancer progression. These findings constitute a first step to explore the molecular mechanisms leading to ER-positive or ER-negative mammary tumors. In addition, we find that ER-positive tumors arising in *Wnt-1* TG mice are refractory to both ovariectomy and the ER antagonist tamoxifen, but lose ER expression with tamoxifen, suggesting that antiestrogen selects for ER-negative tumor cells and that the ER-positive cell fraction is dispensable for growth of these tumors. This is a first report of a mouse model of antiestrogen-resistant ER-positive breast cancers, and could provide a powerful tool to study the molecular mechanisms that control antiestrogen resistance.
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Keywords: estrogen receptor; breast cancer; Wnt; mouse model

Introduction

Breast cancer develops and evolves as genetic and epigenetic alterations accumulate in the ductal epithelium, in which approximately 10–15% of the normal cells express the α isoform of estrogen receptor (ER, Clarke *et al.*, 1997). These changes initially give rise to precursor lesions such as usual or atypical ductal hyperplasias, which may or may not progress to *in situ* and then to invasive breast cancer (Allred *et al.*, 2004). This process of malignant evolution is promoted by the female hormone estrogen. In the end, two distinct types of breast cancer develop – 75% of all breast cancers express ER in a few to nearly 100% of the tumor cells (ER^+) and the rest lack ER entirely (ER^-; Allred *et al.*, 2004). The majority of ER^+ tumors respond initially to antiestrogen therapies despite their high or low level of ER expression, but approximately 30% of them do not and have a poorer prognosis (Allred *et al.*, 2004).

It is not known what determines whether a breast cancer expresses ER, and it is poorly understood why approximately 30% of ER^+ breast cancers do not respond to antihormone therapies (Clarke *et al.*, 2003; Allred *et al.*, 2004). Most studies attempting to address these issues have been carried out using the few available ER^+ human breast cancer cell lines and their xenograft models; however, clinical breast cancer is comprised of heterogeneous cells, including usually both ER^+ and ER^- tumor cells and cells at different stages of differentiation (Dontu *et al.*, 2004). Animal models would be very useful for deciphering the mechanisms leading to ER^+ cancers and to antiestrogen resistance. However, only a few ER^+ models have been made (Medina *et al.*, 1980, 2002; Matsuzawa, 1986; Nandi *et al.*, 1995; Rose-Hellekant *et al.*, 2003; Tilli *et al.*, 2003; Gattelli *et al.*, 2004; Lin *et al.*, 2004; Torres-Arzayus *et al.*, 2004). To our knowledge, ER^+ tumors arising in the few genetically engineered mouse models have not been reported to either respond to or to resist antiestrogen interventions.

Wnts control many developmental processes including mammary morphogenesis and progenitor cell renewal (reviewed by Alonso and Fuchs, 2003; Chu *et al.*, 2004). Made as secreted glycoproteins, Wnts exert their biological effects by binding to their membrane receptors, the frizzled and low-density-lipoprotein receptor-related proteins. As a result, β-catenin is stabilized, translocates to the nucleus and transactivates different genes depending on cellular context. Genes encoding

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components and transcriptional targets of the Wnt signaling pathway are mutated or deregulated in several types of human tumors including breast cancers (Ugolini *et al.*, 2001; Hatsell *et al.*, 2003; Bafico *et al.*, 2004; Brennan and Brown, 2004; Klopocki *et al.*, 2004; Milovanovic *et al.*, 2004).

Wnt-1 (Int-1) was discovered as a gene frequently activated in mammary tumors arising in mice infected with mouse mammary tumor virus (MMTV, Nusse and Varmus, 1982). The MMTV-*Wnt-1* transgenic (TG) model was created by overexpressing *Wnt-1* using its endogenous promoter and the MMTV enhancer elements (Tsukamoto *et al.*, 1988). This unique construct leads to the expression of *Wnt-1* in mammary buds during embryogenesis (Cunha and Hom, 1996). This transgenes appears to cause expansion of mammary progenitor cells, since there is a significant increase in cells expressing putative progenitor cell markers (such as Sca-1 and keratin 6) and cells effluxing fluorescent Hoechst 33342 dye – the dye-excluding property has been associated with stem cells in the hematopoietic system (Goodell *et al.*, 1996; Li Y *et al.*, 2003; Liu *et al.*, 2004). The resulting tumors in MMTV-*Wnt-1* TG mice also seem to arise from progenitor cells because the tumor cells also express Sca-1 and keratin 6, and because they contain heterogeneous tumor cells that share a common genetic mutation in *Pten*, implying a common progenitor (Cui and Donehower, 2000; Rosner *et al.*, 2002; Li Y *et al.*, 2003; Henry *et al.*, 2004; Liu *et al.*, 2004).

In this report, we present evidence to suggest that there is a causal relationship between aberrant Wnt-1 signaling and ER^+ mammary tumors, and that mutant *p53* or overexpressed *Neu*, but not defective *Pten* or activated *Ras*, can suppress the expression of ER in mammary tumors arising in *Wnt-1* TG mice. In addition, we provide evidence to suggest that tumors arising in *Wnt-1* TG mice, although ER^+, are resistant to ovariectomy and to tamoxifen, but lose ER^+ cells with tamoxifen treatment.

Results

ER and PR are expressed in mammary tumors and pulmonary metastases in Wnt-1 *TG mice*

Using a commercial rabbit antibody against the C-terminus of ER-α in immunohistochemical staining, we detected positively stained cells in the majority of mammary tumors and their pulmonary metastases from *Wnt-1* TG mice (ages 3–9 months, Figure 1a). To verify the specificity of this antibody, we also stained normal mammary glands and two human breast cancer cell lines. As expected, staining was detected in heterogeneous patterns in luminal epithelial cells, but not myoepithelial cells, in normal mouse mammary glands; strong staining was detected in MCF7, an ER^+ cell line, but not in CAL51, an ER^- cell line (data not shown). We further confirmed positive staining in these tumors using a second commercial antibody against the N-terminus of ER-α (data not shown). Using both antibodies in Western blotting, we also detected, in all tumors from *Wnt-1* TG mice, a specific band migrating at the same apparent molecular weight (68 kDa) as the stained band of MCF-7-positive control cells (Figure 1b).

A faster-migrating band of approximately 46 kDa was also detected when an antibody against the C-terminus was used (Figure 1b, right panel). Truncated variant messages have been reported in mice (Swope *et al.*, 2002), and an N-terminus-truncated ER variant (46 kDa), sometimes detected in human breast cancer cell lines, has been suggested to have a role in membrane functions of ER (Figtree *et al.*, 2003; Li L *et al.*, 2003). It remains to be determined whether this is a true isoform of ER rather than a degradation product, and, if so, whether it has any biological function in these tumors.

Examining 22 tumors from *Wnt-1* TG mice, we found that 19 expressed ER in greater than 5% of the tumor cells (Figure 1c) and were therefore defined as ER^+. Since tumor cells are heterogeneous in *Wnt-1* TG mice, containing both epithelial and myoepithelial populations, we asked whether ER is exclusively located in epithelial tumor cells using coimmunofluorescent staining. Staining for ER was only detected in cells that also stained for keratin 8 (Figure 1d), a marker for ductal epithelial cells in the breast, but not in cells positive for α-SMA (Figure 1d), a marker for myoepithelial cells. Thus, ER is limited to epithelial tumor cells in *Wnt-1*-induced mammary tumors.

PR is a classical transcriptional target of ER; it is coexpressed in 96% of normal ER^+ mammary cells in mammary glands (Clarke *et al.*, 1997). It is also expressed in 67–80% of ER^+ human breast cancers, and predicts a favorable prognosis (McGuire *et al.*, 1991). Thus, we asked whether PR is expressed in tumors from *Wnt-1* TG mice. Using immunohistochemical staining, we detected PR expression in at least 5% of tumor cells in 82% of tumors from *Wnt-1* TG mice (Figure 1a). These data suggest that the transcriptional function of ER is intact in these tumors.

β-Catenin is a central regulator of Wnt signaling. TG expression of a stabilized mutant of β-catenin induces mammary tumors that bear similarities to tumors in *Wnt-1* TG mice (Imbert *et al.*, 2001; Michaelson and Leder, 2001). To our surprise, however, neither ER nor PR was detected in five tumors from *β-catenin* TG mice (Figure 1a; see Discussion for possible explanations). ER was also lacking (Figure 1a) in all five mammary tumors arising in TG mice overexpressing *c-Myc*, one of the best-characterized targets of the Wnt signaling pathway.

Collaborating genetic alterations in Wnt-1 *TG mice modulate the ER status of the resultant tumors*

Having found that ER is expressed in the majority of the mammary tumors in *Wnt-1* TG mice, we next asked what might modulate the ER status of mammary tumors in *Wnt-1* TG mice. Since it takes a mean time of 6 months for the first mammary tumor to appear in

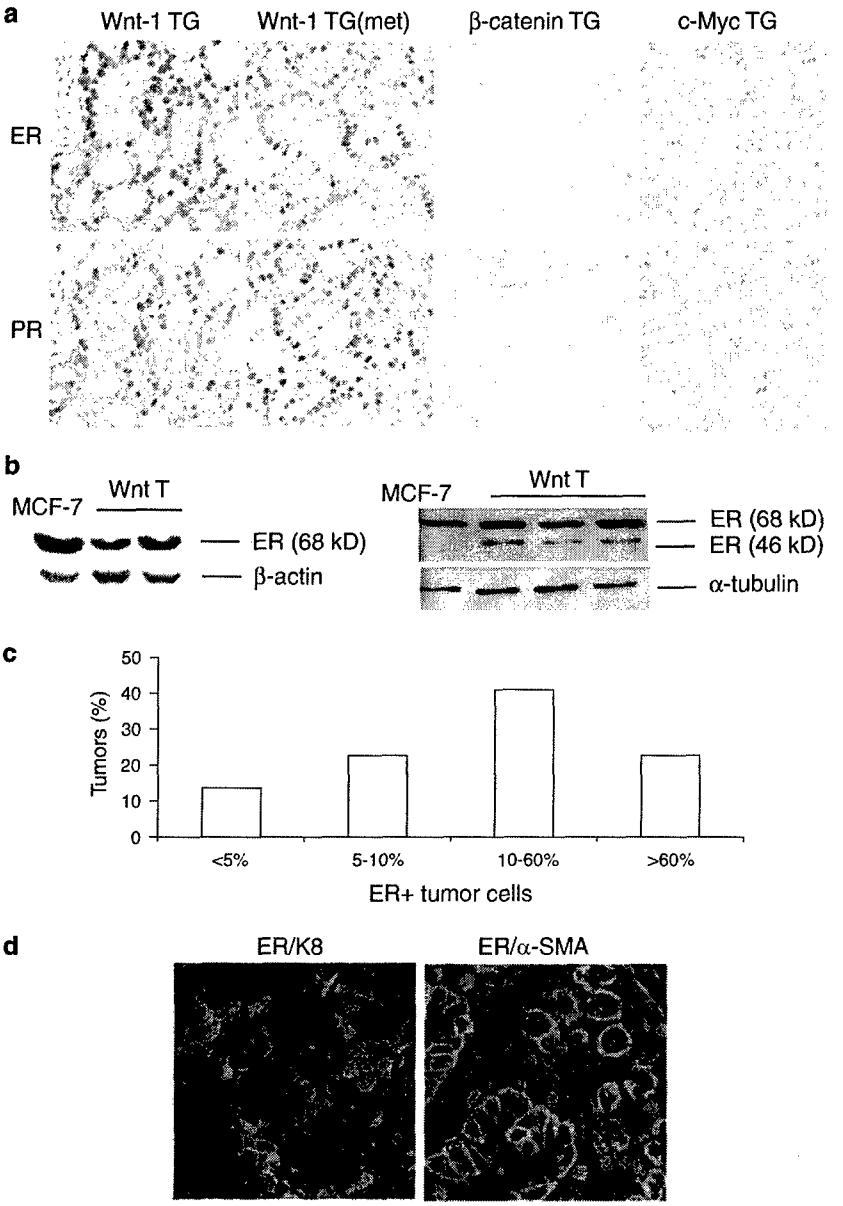

Figure 1 Expression of ER and PR in primary tumors and pulmonary metastases from mice TG for components of the Wnt signaling pathway. (**a**) Immunohistochemical staining for ER and PR in tumors and metastases of tumors from mice carrying the indicated transgene. A ×20 objective was used. (**b**) Western blotting for ER in tumor lysates from *Wnt-1* TG mice. Lysates from MCF-7 were included as a positive control. Staining for α-tubulin or β-actin was included as a loading control. (**c**) Distribution of ER^+ cells in tumors from *Wnt-1* TG mice as determined by counting the percentage of immunohistochemically stained cells. (**d**) Photomicrograph of coimmunofluorescent staining for ER and keratin 8 (K8) and for ER and α-SMA in mammary tumors from *Wnt-1* TG mice. ER is labeled in red, K8 or α-SMA in green. A ×40 objective was used

one of the 10 mammary glands in *Wnt-1* TG mice (Tsukamoto *et al.*, 1988), additional genetic mutations are most likely required to form mammary tumors. Thus, we hypothesized that these additional genetic alterations might affect the ER status of the resulting tumors. We and others have reported several collaborating genetic changes in tumorigenesis in *Wnt-1* TG mice, including mutations of *H-Ras*, loss of *Pten* or *p53*, and overexpression of *Neu* (Donehower *et al.*, 1995; Li *et al.*, 2000, 2001; Podsypanina *et al.*, 2004). When tumors that bore a *Ras*-activating mutation (G12E, G13V, Q61L and Q61R), determined by sequencing tumor DNA, were compared with those that did not, both ER and PR levels were similar by Western blotting (Figure 2a). Therefore, activation of *Ras* does not suppress ER expression in mammary tumor cells that already overexpress *Wnt-1*. However, we were surprised to find that *Ras*-mutated tumors in *Wnt-1* TG mice do

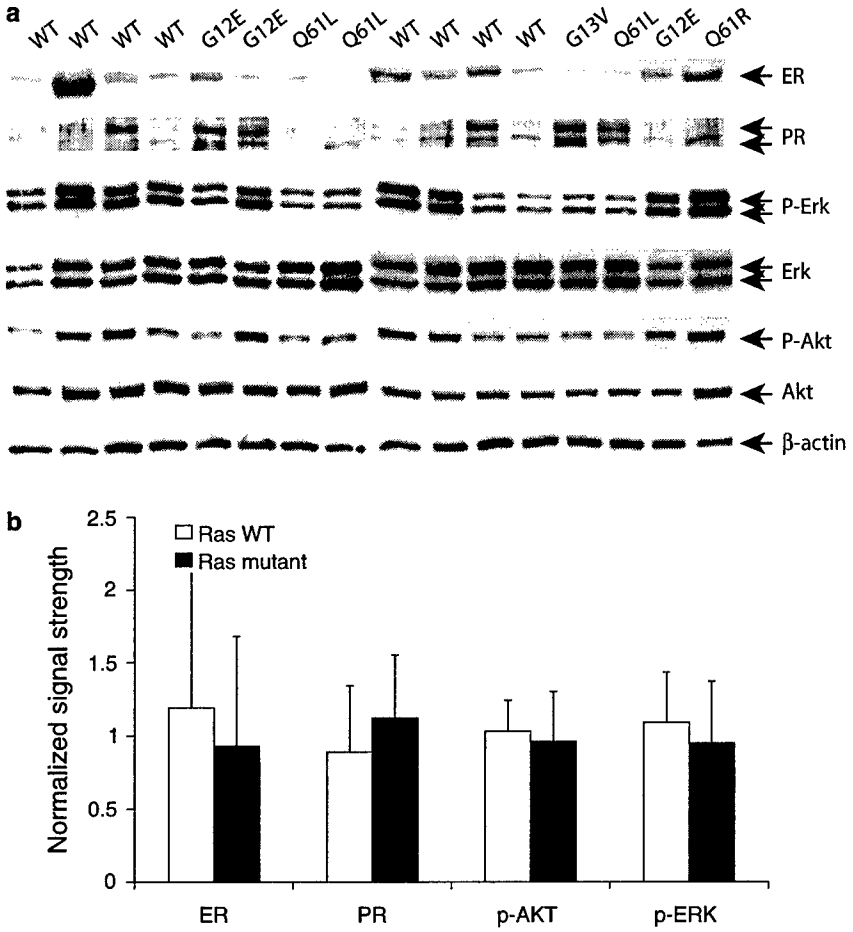

Figure 2 *Ras* status does not affect ER expression in tumors in *Wnt-1* TG mice. (**a**) Protein lysates of mammary tumors with *H-Ras* status indicated at the top were assayed by Western blotting for the proteins indicated at the right. (**b**) Quantitative presentation of levels of the four proteins depicted in (**a**). Normalized signal strength was obtained by dividing the densitometric signal of ER and PR by that of β-actin, and by dividing the signal of P-Akt and P-Erk by that of Akt and Erk, respectively

not make more activated Erk (a classical downstream factor of Ras) or more activated Akt (another downstream target of Ras) than tumors without *Ras* mutations (Figure 2a and Podsypanina *et al*., 2004), since overexpression of mutated H-*Ras* from the MMTV promoter in TG mice or in cultured cells activates Erk signaling (Sinn *et al*., 1987; Podsypanina *et al*., 2004). Similar lack of activation of these two *Ras* effectors has been reported in tumorigenesis of hematopoietic cells, induced by expressing mutated *K-Ras* from its endogenous promoter in mice (Braun *et al*., 2004). Thus, activation of Erk and Akt *in vivo* may require higher levels of mutated *H-Ras* that are only achievable by a stronger promoter, such as that of MMTV. Consequently, it is an open question whether activation of Erk or Akt will cause *Wnt-1*-overexpressing mammary cells to evolve into ER^- or ER^+ tumors.

Pten is mutated in approximately 5% of human breast cancers, and is downregulated in 35–50% of cases (reviewed by Sansal and Sellers, 2004). Loss of *Pten* is usually associated with ER^- human breast cancers (Garcia *et al*., 1999; Perren *et al*., 1999; Shi *et al*., 2003). We have previously reported that inactivation of one allele of *Pten* accelerates mammary tumorigenesis in MMTV-*Wnt-1* TG mice, and 70% of the resulting tumors have lost the wild-type allele of *Pten* (Li *et al*., 2001). To determine if loss of *Pten* alters the ER status of tumors in *Wnt-1* TG mice, we examined preneoplastic and tumor samples from a cross of *Wnt-1* TG mice and *Pten* heterozygous mice (Podsypanina *et al*., 1999). Similar to the staining results from mammary glands from adult non-TG and *Wnt-1* TG mammary glands, ER and PR were detected in hyperplasias in *Wnt-1*/*Pten*+/− mice (Figure 4i and j). Full-blown tumors that had not undergone LOH at the *Pten* locus also retained ER and PR (data not shown). Furthermore, ER and PR were still expressed in four of the five tumors that had lost the wild-type *Pten* allele (Figure 3a and b). These results suggest that loss of *Pten* does not prevent ER expression in mammary cancers, consistent with another study examining ER expression in mammary lesions in *Pten*+/− mice that do not carry a transgene (Shi *et al*., 2003).

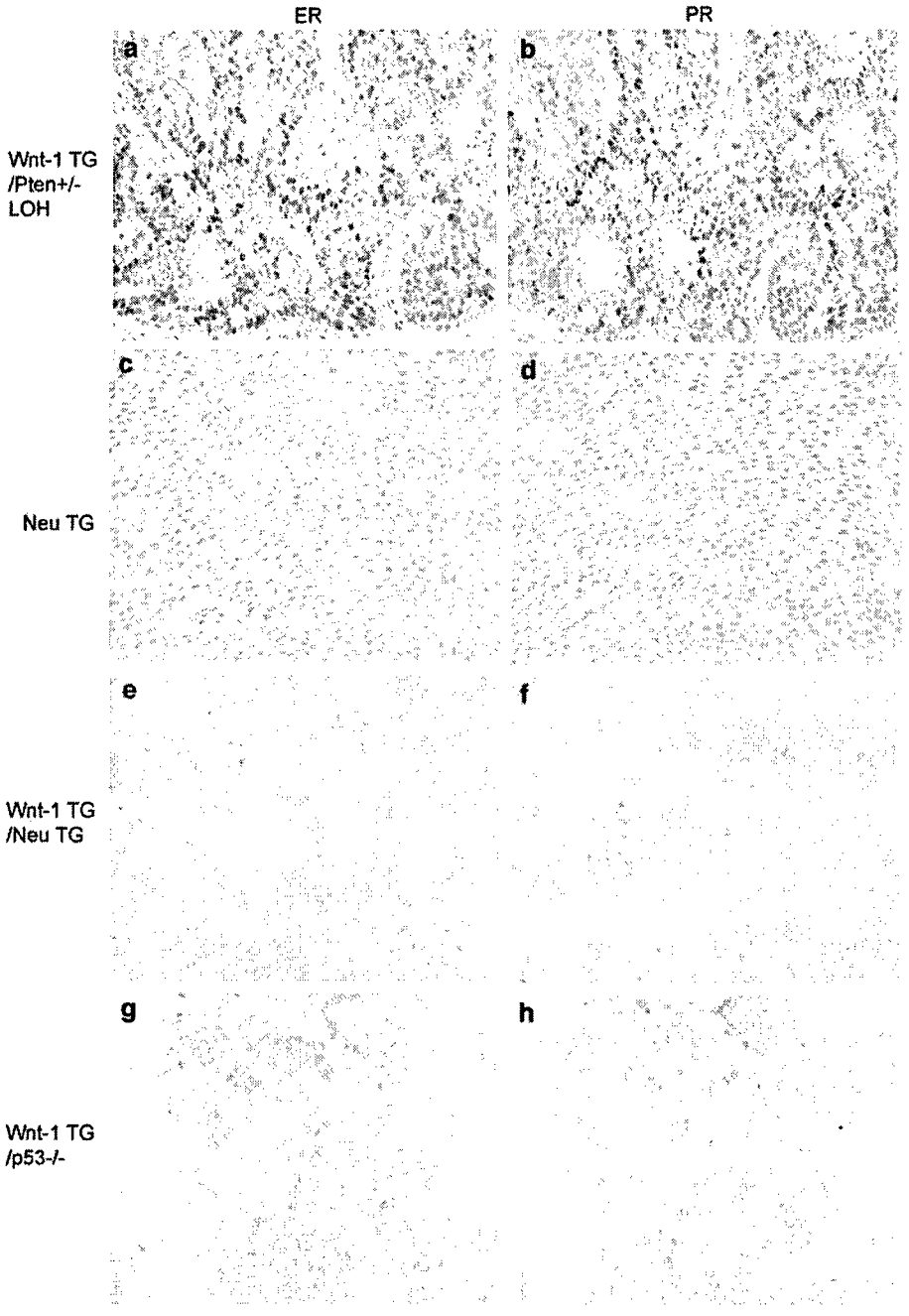

Figure 3 *Neu* overexpression or loss of *p53*, but not loss of *Pten*, leads to ER-/PR- mammary tumors in *Wnt-1* TG mice. Paraffin sections of mammary tumors from mice with the genotypes shown at the left were stained by immunohistochemical staining for the protein indicated at the top. Staining of mammary tumors from MMTV-*Neu* was included as a control. A × 20 objective was used

Neu (HER2, ErbB2) is a member of the epidermal growth factor receptor (EGFR) family of transmembrane tyrosine kinases (Olayioye *et al*., 2000). Its gene is amplified in approximately 25% of human breast cancers (Slamon *et al*., 1987), and is inversely correlated with ER and PR (Konecny *et al*., 2003), but the mechanism underlining this correlation is not known. We have recently reported that the *Neu* protooncogene collaborates with *Wnt-1* in mammary tumorigenesis (Podsypanina *et al*., 2004), and that it remains wild type in tumors arising in animals bi-TG for *Wnt-1* and *Neu* (Podsypanina *et al*., 2004), although it is somatically mutated to a more activated form in approximately 70% of tumors arising in MMTV-*Neu* TG mice that are otherwise wild type (Siegel *et al*., 1994). Therefore, we asked whether ER is retained in preneoplastic and tumor lesions in bi-TG mice expressing both *Wnt-1* and the *Neu* protooncogene. Similar to what was observed in

Wnt-1 or *Neu* TG mice (Figure 4c–f, and reference Wu *et al*., 2002), both ER and PR were present in hyperplastic ducts, albeit heterogeneously, in nontumor-bearing mammary glands from *Wnt-1*/*Neu* bi-TG mice (Figure 4g and h). However, similar to tumors from *Neu* TG mice (Figure 3c and d and reference Wu *et al*., 2002), but different from tumors from *Wnt-1* TG mice, neither ER nor PR was detected in any of the four tumors from these bi-TG mice (Figure 3e and f). Therefore, *Neu* prevents *Wnt-1*-expressing mammary tissues from evolving into ER^+ tumors.

P53 is mutated in approximately 50% of human breast cancers, and the pathway is probably disabled in even higher percentages of cases (Gasco *et al*., 2002).

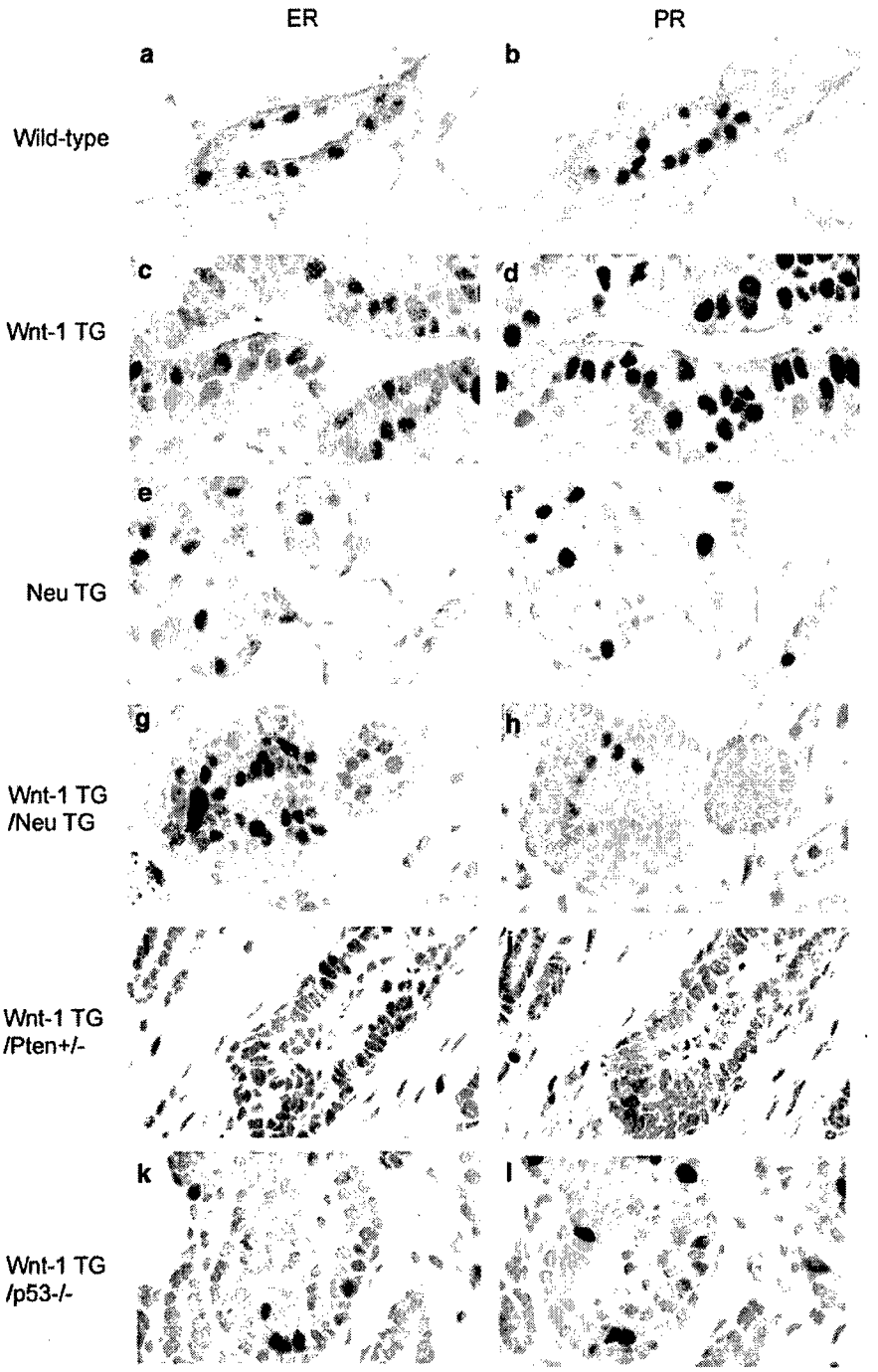

Figure 4 ER and PR are expressed in mammary ductal hyperplasias in TG mice. Paraffin sections of mammary glands from adult mice (12 weeks old) with the genotypes shown at the left were stained by immunohistochemical staining for the protein indicated at the top. A ×20 objective was used

Loss of *p53* does not inhibit the expression of ER in normal mammary cells, since ER is expressed in *p53*-null mammary epithelia (data not shown and Medina *et al.*, 2003). Loss of *p53* does not have a significant correlation with the ER status of human breast tumors, and 20% of tumors arising in mammary epithelia from *p53*-knockout mice express ER (Barbareschi *et al.*, 1996; Medina *et al.*, 2002). To determine if germline *p53* loss might modulate the expression of ER in *Wnt-1* TG mice, we examined both preneoplastic and tumor sections from mice that were *Wnt-1* TG/*p53*−/−. While retained in intraductal hyperplasias in these mice (Figure 4k and l), both ER and PR were undetectable in all five tumors we examined by immunohistochemical staining (Figure 3g and h) and Western blotting (data not shown). These results suggest that loss of *p53*, like activated Neu signaling, inhibits *Wnt-1*-expressing mammary tissues from evolving into ER$^+$ tumors.

Wnt-1-*induced mammary tumors continue to grow when estrogen signaling is inhibited*

Approximately 70% of ER$^+$ human breast cancers are growth inhibited by antihormone therapies, but the rest are not (Allred *et al.*, 2004). The mechanisms leading to antihormone responsiveness are poorly understood (Clarke *et al.*, 2003). Although activated Wnt signaling might be associated with a subset of ER$^+$ human breast cancers, based on the correlation between reduced expression of secreted frizzled-related protein 1 (an inhibitor of Wnt signaling) and expression of ER (Ugolini *et al.*, 2001), it is not known whether these ER$^+$ cancers are susceptible to antiestrogens. It has been reported that Wnt-1 can induce mammary tumors in mice that lack ER (Bocchinfuso *et al.*, 1999); however, it is not known whether ER signaling is necessary for survival of the ER$^+$ tumors that develop in ER-intact *Wnt-1* TG mice. Therefore, we transplanted three ER$^+$ mammary tumors from *Wnt-1* TG mice into 15 nude mice each. When the tumors reached 0.7 cm in diameter, we divided the nude mice in each group into three sets and administered ovariectomy, daily tamoxifen or daily vehicle. We measured tumor size weekly and euthanized all recipient mice 4 weeks later. Both ovariectomy and tamoxifen failed to prevent or inhibit tumor growth (Figure 5a), although the same dose of tamoxifen successfully inhibited the growth of control ER$^+$ tumors in these mice resulting from transplantation with MCF-7 cells (data not shown). ER and PR were detected in the transplanted tumors before and after treatment with the vehicle control (Figure 5b and c), eliminating the trivial explanation that the transplants do not have functional ER due to the lower circulating estrogen levels sometimes found in nude mice. Thus, we conclude that estrogen signaling is dispensable for growth of MMTV-*Wnt-1*-induced ER$^+$ tumors.

One of the mechanisms leading to estrogen-independent growth is that the transcriptional activity of ER no longer depends on its ligand, due to phosphorylation of ER (Le Goff *et al.*, 1994; Kato *et al.*, 1995; Pietras *et al.*, 1995; Shang and Brown, 2002; Michalides *et al.*, 2004) or to stabilization of its interaction with coactivators (Trowbridge *et al.*, 1997; Zwijsen *et al.*, 1997). However, we found that both ER and its transcriptional target PR were expressed in far fewer cells in tumors from mice treated with tamoxifen than in control tumors or in donor tumors prior to transplantation (Figure 5b and c), suggesting a different mechanism of resistance. The diminished ER expression must be due either to downregulation of ER expression in ER$^+$ tumor cells or to selection against the ER$^+$ subset of tumor cells. With regard to the first possibility, we found that tamoxifen did not downregulate ER in the adjacent normal ducts (data not shown), suggesting that the loss of ER in treated tumors is not caused by downregulation of ER expression by tamoxifen, consistent with reports in cultured breast cancer cell lines (Giamarchi *et al.*, 2002; Cheng *et al.*, 2004). To confirm this in the tumor cells themselves, we examined ER expression in three sets of transplanted tumors that had been treated with tamoxifen for only 5 days, when the ER$^+$ cell population would not yet be selected out. As expected, the percentage of ER$^+$ cells did not change in that period (data not shown). Thus, we conclude that the antiestrogen resistance of ER$^+$ mammary tumors in *Wnt-1* TG mice probably does not result either from ligand-independent transactivation by ER or from suppression of ER expression in ER$^+$ tumor cells, but rather from selection against the ER$^+$ population, leaving the ER$^-$ cells actively growing.

Discussion

In this study, we demonstrate that TG expression of *Wnt-1* in the mammary gland induces largely ER$^+$ mammary tumors, while loss of *p53* or overexpression of *Neu*, but not activating mutations of *Ras* or loss of *Pten*, lead to ER$^-$ tumors in these *Wnt-1* TG mice. In addition, we show that tumors arising in *Wnt-1* TG mice continue to grow in spite of ovariectomy or tamoxifen, possibly through selection against ER$^+$ tumor cells.

While rare in most tumors induced by other oncogenes or defective tumor suppressor genes, ER$^+$ tumors predominate in *Wnt-1* TG mice. ER signaling might facilitate Wnt signaling in transforming mammary cells – in fact, ectopic activation of estrogen signaling has been reported to enhance the transcriptional activities of the Wnt signaling intermediate β-catenin in colon cancer cell lines (Kouzmenko *et al.*, 2004). However, it appears that ER signaling is not absolutely required for *Wnt-1* to induce mammary tumors. First, mammary tumors have been observed in male *Wnt-1* TG mice (Tsukamoto *et al.*, 1988) and in females that have lost exon 1 of *ER* (but retained the expression of a 61-kDa truncated ER isoform), although these tumors occur much later (Bocchinfuso *et al.*, 1999). Second, a small percentage of mammary tumors arising in *Wnt-1* TG mice are actually ER$^-$ (Figure 1c). Last, there are a great many ER$^-$ cells even in ER$^+$ hyperplasias and tumors in *Wnt-1* TG mice.

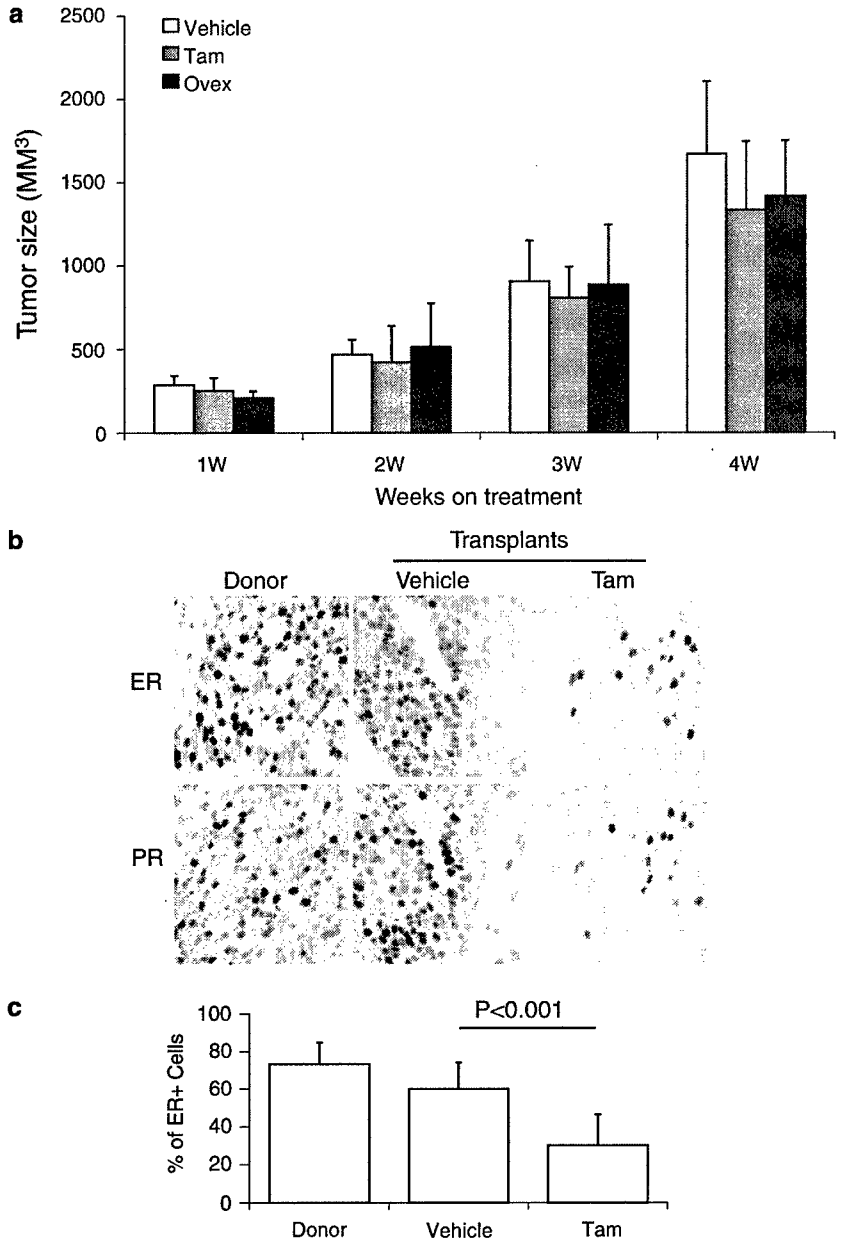

Figure 5 Ovariectomy and tamoxifen do not suppress growth of transplanted mammary tumors from MMTV-*Wnt-1* TG mice, but do reduce the frequency of ER$^+$ cells. (**a**) Tamoxifen or ovariectomy does not suppress the growth of mammary tumors from *Wnt-1* TG mice. Each of three independent MMTV-*Wnt-1*-induced tumors was transplanted into 15 nude mice (2 mm^3 each recipient). When the transplanted tumors reached 0.7 cm in diameter, the mice in each group were divided into three subgroups, which were treated with ovariectomy (ovex), tamoxifen (tam), or vehicle for 4 weeks. (**b**) ER and PR are suppressed by antiestrogenic therapies. Transplanted tumors were collected after 4 weeks of treatment and stained for the proteins indicated at the left. Photomicrographs are representative of three independent antiestrogenic treatment experiments. (**c**) Percent cells positive for ER in donors and in transplanted groups after 4 weeks of treatment. Quantitative data were generated by counting positively stained cells in nine tam-or vehicle-treated tumors (three transplants from each of the three donors). Five independent $\times 40$ microscopic fields were counted per section per tumor. *P*-value is shown

Then, what accounts for the predominance of ER$^+$ tumors in *Wnt-1* TG mice? We and others have reported evidence that activated Wnt signaling might induce tumors from progenitor cells. If Wnt signaling, which is known to regulate progenitor cell renewal and proliferation, does cause mammary tumors from progenitor cells, the transformed progenitor cells in tumors might still retain the ability to both self-renew and differentiate into multiple cells including both ER$^-$ and ER$^+$ cells, therefore resulting in ER$^+$ tumors. Indeed, *Wnt-1*-

induced tumors contain a small subset of cells that bear similarities to normal progenitor cells, based on staining for putative progenitor cell markers and detection of cells that exclude the DNA-binding dye Hoechst 33342 (Li Y *et al.*, 2003; Liu *et al.*, 2004). The rest of the tumor cells appear to be differentiated epithelial and myoepithelial cells. The presence of a small subset of tumor cells (tumor progenitor cells or cancer stem cells) that can self-renew and differentiate into cells of multiple lineages has been reported for several tissue types, including the breast (Pardal *et al.*, 2003; Singh *et al.*, 2004), although the cellular origin of these cells or their hosting tumors has not been revealed.

Wnt signaling activates several downstream pathways. The best studied, the so-called 'canonical' pathway, leads to stabilization of β-catenin, which in turn forms heterodimers with members of the LEF/TCF family of DNA binding proteins and transactivates a number of transcriptional targets, including *c-Myc* and *Cyclin D1* (Hatsell *et al.*, 2003; Nusse, 2003; Brennan and Brown, 2004). However, to our surprise, neither ER nor PR was detected in tumors from mice TG for *β-catenin* or *c-Myc* (Figure 1a). The Wnt-1 induction of ER^+ tumors, possibly through regulating progenitor cell self-renewal and multilineage differentiation, may require paracrine Wnt signaling or β-catenin-independent noncanonical Wnt signaling through Rho, JNK and PKC (Hatsell *et al.*, 2003). However, we cannot exclude the possibility that the tumors in *β-catenin* TG mice arose from a slightly different (probably more differentiated) population of mammary cells due to the use of a different TG promoter (Li Y *et al.*, 2003).

The loss of *Pten* does not abolish the expression of ER in tumors in *Wnt-1* TG mice (Figure 3a). It has been reported that ER is retained in breast lesions arising in *Pten* heterozygous mice that are otherwise wild type (Shi *et al.*, 2003), although the authors did not indicate whether the wild-type allele of *Pten* was lost in the epithelial cells in their lesions. Jointly, these data suggest that *Pten* loss may not suppress ER expression in mammary tumors or prevent transformed progenitor cells from differentiating into ER^+ cells. Interestingly, Akt – which is usually phosphorylated and activated in other cell types when *Pten* is suppressed, and has been reported to suppress ER expression in cultured breast cancer cells (Faridi *et al.*, 2003) – is not highly phosphorylated in mouse tumors that are *Wnt-1* TG/*Pten* +/− with LOH (Li *et al.*, 2001), similar to what has been reported in some human breast cancers that have downregulated *Pten* (Shi *et al.*, 2003). Therefore, our data do not suggest that activation of Akt during the evolution to tumors in *Wnt-1* TG mice leads to ER^+ tumors.

Although *H-Ras* is mutated in approximately 50% of tumors arising in *Wnt-1* TG mice (Podsypanina *et al.*, 2004), these *H-Ras* mutants do not have detectable effects on either the expression of ER or its function in inducing PR in these tumors (Figure 2). Thus, *H-Ras* mutants most likely do not suppress ER expression in breast cancer or prevent transformed progenitor cells from differentiating into ER^+ cells. This *in vivo* study is consistent with an earlier cell culture study, in which mutant *Ras* was found not to inhibit the expression of ER in MCF-7 cells (Sukumar *et al.*, 1988). Interestingly, ER is not expressed in mammary tumors induced by TG overexpression of an *H-Ras* mutant with no pre-existing aberration of Wnt signaling (data not shown). In addition to possible differences in cellular origin between ER^+ *Ras*-mutated tumors in *Wnt-1* TG and ER^- tumors in *H-Ras* TG mice (Li Y *et al.*, 2003), there are other important dissimilarities between these two tumors, including levels of *H-Ras* expression and levels of activation of the Erk and Akt effector pathways (Podsypanina *et al.*, 2004). Thus, it remains to be determined whether ER^- tumors will form in *Wnt-1* TG mice if *H-Ras* mutants are expressed from a stronger exogenous promoter during the evolution to mammary tumors.

It is striking that either overexpression of *Neu* or loss of *p53* causes exclusively ER^- tumors in *Wnt-1* TG mice. Several mechanisms may lead to altered ER status, including the cellular origin of cancer, direct modulation of ER expression by these secondary genetic factors and alteration of differentiation of transformed progenitor cells. It is plausible that additional alteration in Neu signaling and p53 functions may select different cells in the mammary gland to form cancer. However, the ER^- tumors in *Wnt-1* TG mice that either overexpress *Neu* or lose *p53* seem to arise from the same cells as the ER^+ tumors in *Wnt-1* TG mice, that is, the progenitor cells (Li Y *et al.*, 2003). First, they contain both myoepithelial tumor cells and epithelial tumor cells, implying an origin from a bipotential cell (Li Y *et al.*, 2003; Podsypanina *et al.*, 2004; and K Podsypanina, unpublished). Second, they harbor tumor cells expressing the putative progenitor cell markers keratin 6 and Sca-1 (Li Y *et al.*, 2003; Podsypanina *et al.*, 2004). Last, the microarray gene expression profiles are remarkably similar between tumors from *Wnt-1* TG/*p53*−/− mice and tumors from *Wnt-1* TG mice that are otherwise wild type (S Huang, unpublished). Collectively, these results argue against different cellular origins for these tumors.

It is also unlikely that lack of ER expression in tumors in *Wnt-1* TG mice that either overexpress *Neu* or lose *p53* is the consequence of direct suppression of ER by inactivation of *p53* or overexpression of *Neu*. As stated above, ER-expressing cells are present in preneoplastic lesions in mammary glands in both *Wnt-1* TG/*p53*−/− and *Wnt-1*/*Neu* bi-TG mice (Figure 4), suggesting that these lesions do not directly suppress ER in *Wnt-1*-overexpressing cells. However, we cannot exclude the possibility that they may be able to suppress ER after these cells have evolved into cancer, or that other genetic events (such as ErbB3 activation; Siegel *et al.*, 1999) that are preferentially selected for during tumorigenesis in these compound mutant cells may inhibit ER in cancer cells.

Since *Wnt-1* appears to induce mammary tumors from progenitor cells and the transformed progenitor cells can both self-renew and differentiate into both ER^- and ER^+ tumor cells, it is possible that acquisition of another genetic alteration, such as overexpression of

Neu or ablation of *p53*, might impair the potential of these tumor progenitor cells to differentiate into ER^+ cells, thus leading to ER^- tumors (even though, in precancerous stages, these genetic defects do not seem to totally prevent cancer precursor cells from differentiating into ER^+ cells). Consistent with this hypothesis, tumors arising in *Wnt-1* TG/*p53*−/− mice have been reported to exhibit less differentiation than tumors arising in *Wnt-1* TG/*p53*+/+ mice (Donehower *et al.*, 1995); the signaling network activated by *Neu* has been reported to have a role in regulating cell differentiation (Dai and Holland, 2003). To test whether *Neu* or defective *p53* indeed suppresses the differentiation of cancer progenitor cells in these models, we plan to inhibit Neu signaling or restore *p53* in these ER^- tumors to test whether removing these genetic events can permit the ER^- tumor progenitor cells to differentiate into ER^+ cells.

The likely presence of transformed tumor progenitors in *Wnt-1* TG mice might also explain why these ER^+ tumors are resistant to antiestrogens. If *Wnt-1* transforms ER^- progenitor cells, the differentiated ER^+ progeny cells might be dispensable for tumor growth. A similar hypothesis has been proposed in a recent review article (Dontu *et al.*, 2004) to explain human tumor resistance to antiestrogen therapies. We are currently testing this hypothesis in several mouse models, and additionally asking whether antiestrogen resistance in *human* ER^+ cancers might also result from lack of ER expression in the cancer progenitor cells.

In conclusion, activation of Wnt-1 signaling induces ER^+ mammary tumors, but additional genetic mutations can alter the ER status of the resultant tumors, possibly through restricting differentiation of tumor progenitor cells. These ER^+ tumors are resistant to antiestrogens, probably because the ER^- fraction of tumor cells can maintain tumor growth in the absence of estrogen signaling.

Materials and methods

Mice

TG mice expressing *Wnt-1* (Tsukamoto *et al.*, 1988), the *Neu* protooncogene (Guy *et al.*, 1992), an N-terminus-deleted, stabilized mutant of *β-catenin* (Imbert *et al.*, 2001) and *c-Myc* (Stewart *et al.*, 1984) have been described, as have mice harboring targeted inactivating mutations of *Pten* (Podsypanina *et al.*, 1999) and *p53* (Jacks *et al.*, 1994). All TG mice were maintained on the FVB background, but *Pten* and *p53* mutant mice were on mixed backgrounds of 129, FVB and C57/BL6. All mice were housed in specific-pathogen-free animal facilities, on a standard diet. Genotyping was carried out using PCR of tail DNAs (Tsukamoto *et al.*, 1988).

Antibodies

The following antibodies were used: purified rabbit antibodies against the ER C-terminus (Santa Cruz, #SC542) or the N-terminus (Novocastra, #NCL-ER-6F11), progesterone receptor (PR, DAKO, #A0098 for immunohistochemical staining; Santa Cruz , #SC-7208 for Western blotting), phospho-AKT (ser473, Cell Signaling, #9102), Akt (Cell Signaling, #9272), phospho-Erk (Cell Signaling, #9101), Erk (Cell Signaling, #9102) and β-actin (Sigma, #5441); purified mouse monoclonal antibodies against α-smooth muscle actin (α-SMA, DAKO) and bromodeoxyuridine (BrdU, Becton-Dickinson, #347580); and partially purified rat antibodies against keratin 8 (Kemler *et al.*, 1981), purchased from the Developmental Studies Hybridoma Bank organized under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA.

Immunoassays

Tissues were fixed in 10% neutral formalin and processed as described previously (Li *et al.*, 2001) to obtain paraffin sections of 4 μm in thickness. For immunohistochemistry, the sections were boiled for 15 min in citrate buffer, pH 6.0 (to unmask antigen epitopes). Endogenous peroxidase activity was inactivated by a 10 min incubation in 3% hydrogen peroxide, and subsequent steps were performed using Vector ABC and MOM kits and the Novo-Red substrate (Vector Laboratories) following the manufacturer's recommendations. To label cells in S phase of the cell cycle, BrdU (Sigma, #B-5002) at 100 μg/g of mouse body weight in saline was injected intraperitoneally 1 h prior to euthanasia.

For Western blotting, tumors were ground to powder in liquid nitrogen and lysed in the M-PER tissue lysis solution (Pierce) with gentle shaking overnight at 4°C. The resulting supernatant (25 μg protein) was denatured using 2-mercaptoethanol, resolved on 10% polyacrylamide minigels containing 10% SDS and transferred onto nitrocellulose membranes, which were then incubated with primary antibodies and peroxidase-conjugated secondary antibodies (Jackson Laboratory) in tris-borate buffer/0.05% Tween-20/5% nonfat dried milk. The proteins recognized by the specific antibodies were visualized by a chemiluminescent substrate (Supersignal, Pierce).

Analysis of H-Ras *mutations in tumors from* Wnt-1 *TG mice*

The method has been described (Podsypanina *et al.*, 2004). Briefly, DNA was extracted from tumors from *Wnt-1* TG mice and used for PCR amplification of exons 1 and 2 of *H-Ras* using two sets of oligos (mouse *H-Ras* exon 1: HRAS.F1A – 5′-CCTTGGCTAAGTGTGCTTC-3′, HRAS.B1A – 5′-CCA CCTCTGGCAGGTAG-3′; mouse *H-Ras* exon 2: HRAS.F2A – 5′-GGATTCTCTGGTCTGAGG-3′, HRAS.2B2 – 5′-GGA TATGAGCCAGCTAGC-3′). The resulting PCR product was purified and sequenced to identify mutations in these two exons. We have reported earlier that only exons 1 and 2 of *H-Ras* are mutated in *Wnt-1*-induced mammary tumors; other exons or family members of *Ras* are normal in these tumors (Podsypanina *et al.*, 2004).

Tumor transplantation and antiestrogen treatment

Tumors of 1.5 cm in diameter were excised from *Wnt-1* TG mice, minced into cubes of 2 mm in size and injected subcutaneously using a trocar into female nude mice of 8 weeks of age. In all, 15 nude mice were transplanted for each donor tumor from *Wnt-1* TG mice. When transplanted tumors reached 0.7 cm in diameter, the recipient mice were divided into three groups and given ovariectomy, tamoxifen or vehicle. Tamoxifen (Sigma), dissolved in peanut oil at a concentration of 10 mg/ml, was injected subcutaneously five times a week (0.5 mg/mouse). Tumor size were measured weekly. At 4 weeks after the initiation of treatments, all mice were killed, and tumors were collected for immunohistochemical staining for ER and PR.

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Stem/Progenitor Cells in Mouse Mammary Gland Development and Breast Cancer

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Breast cancer is a genetically and clinically heterogeneous disease. It is unclear whether different target cells contribute to this heterogeneity and which cell types are most susceptible to oncogenesis. Stem cells are speculated to be the cellular origin of at least a subset of human breast cancers. To begin to address these issues, we have isolated and characterized cell populations enriched in normal mammary stem/progenitors and have studied the expression of putative stem/progenitor markers in tumors derived from genetically engineered mice. Specifically, transgenic activation of Wnt signaling in the mammary gland induces tumors comprised of epithelial and myoepithelial cells harboring the same genetic defect implying that the tumor arose from transformation of a bipotent progenitor cell. On the other hand, transgenic activation of Neu signaling induces tumors comprising cells of more limited lineage capacity. Thus, the heterogeneity of different breast cancers may reflect the activation of different oncogenic pathways, different cellular targets in which these genetic changes occur, or both.

KEY WORDS: mammary gland; stem/progenitor cells; breast cancer; Wnt; ErbB2/HER2/Neu.

INTRODUCTION

The existence of adult mammary stem cells was established several decades ago when DeOme and his colleagues *(1)* observed that epithelium isolated from several different regions of mammary gland was able to generate normal mammary outgrowths containing ductal, alveolar, and myoepithelial cells. Further transplantation studies by Smith and Medina *(2)* demonstrated that these stem cells existed throughout the life-span of the mammary gland. Limiting dilution transplantation experiments using mammary epithelial cells tagged with the mouse mammary tumor virus (MMTV) indicated that clonal progenitors were capable of generating complete, functional, mammary outgrowths when transplanted into the cleared mammary fat pads of recipient mice *(3,4)*. Estimates of the frequency of these cells range from 1 per 1000 to 1 per 2000 mammary epithelial cells, and it has been suggested that the proportion of these cells remains relatively constant throughout mammary gland development *(5)*. Thus, mammary stem cells must undergo both symmetric as well as asymmetric cell division, since the total number of mammary epithelial cells expand dramatically during pregnancy.

It has been speculated that stem cells may represent the cellular origin of cancer, since they exist quiescently over long periods of time, and could, therefore, accumulate multiple mutations over the life-span of an organism, ultimately giving rise to tumors when stimulated to proliferate *(6)*. Women

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Abbreviations used: SP, side-population; Sca-1, stem cell antigen-1; MMTV, mouse mammary tumor virus; LRC, label-retaining cell; BCRP1, breast cancer resistance protein-1; MEC, mammary epithelial cell; TEB, terminal end bud; PyMT, polyoma middle T antigen; WAP, whey acidic protein.

exposed as teenagers to ionizing radiation are more susceptible to breast cancer than those exposed as adults, and these women developed breast cancer several decades following their initial exposure *(7)*. These results, as well as the observation that almost 40% of breast cancers recur after 10 years following the diagnosis and removal of the primary tumor *(8)*, suggest that a population of cells exists with an extremely long half life, similar to stem cells, which may be important in the etiology of breast cancer.

Highly tumorigenic cells with properties consistent with those of stem/progenitor cells have been isolated from cancers of several tissue types *(9,10)*. Recently, such cells have been identified from human breast cancers *(11)*. Transplantation of as few as several hundred of these cells, but not other cells from these cancers, into mammary fat pads of NOD/SCID etoposide-treated mice recapitulated the original tumors *(11)*. These data suggest that cancer stem cells may exist in human breast cancer. This has lead to the hypothesis that conventional therapies might not successfully remove all of these tumor stem cells, possibly as a consequence of the increased expression of members of the ABC family of drug transporters. This may ultimately result in recurrence or metastasis even when remarkable initial responses are observed clinically *(12)*.

The following discourse presents a brief overview of the characterization of normal mammary stem/progenitors and their role in the etiology of a number of different genetically engineered mouse models of mammary cancer, with an emphasis on the studies from our own laboratories.

ISOLATION AND CHARACTERIZATION OF NORMAL MAMMARY STEM/ PROGENITOR CELLS

Several methods are currently being utilized to isolate and study putative mammary stem/progenitor cells, including long-term bromodeoxyuridine (BrdU) labeling to identify label-retaining cells (LRCs), Hoechst dye efflux to identify side population (SP) properties, and potential stem cell-cell surface markers, such as stem cell antigen-1 (Sca-1, 13). In the hematopoietic system, cells that efflux Hoechst 33342 dye and segregate into a spur or "side-population" on flow cytometry analysis have been shown to represent a small fraction of the bone marrow. The efflux of the Hoechst dye is due to the existence of the BCRP1 (breast cancer resistance protein-1)/ABCG2 transporter on the SP cells (see below). These cells are capable of recapitulating the bone marrow in irradiated mice, establishing their functional capacity as hematopoietic stem cells *(14)*. A comparable SP population derived from mammary epithelial cells also has been demonstrated in both the mouse and human mammary glands *(13, 15–17)*. Human SP cells have been shown to comprise about 5% of human breast epithelial cells defined by BER-EP4. They are enriched for ERα/PR+ cells relative to non-SP cells, and they co-express putative stem cell markers, including p21, cytokeratin 19, and Musashi, a translational regulator important in controlling asymmetric cell division in stem cells *(17,18)*. Other studies have identified a bipotent, $K18^+/K14^+$ primitive precursor population in SP cells *(16)*.

The BCRP1 pump was first identified in breast cancer cells resistant to topoisomerases and may be responsible for the mechanism of drug resistance in many types of cancer *(19)*. An increase in SP cell survival following chemotherapy has been attributed to the expression of ABC transporters that have high drug-efflux capacity, including ABCG2 (BCRP1) and ABCA3, as highlighted by a recent study in solid tumors *(20)*. Interestingly, the BCRP1 pump has been demonstrated to confer a survival advantage to hypoxic SP cells not through its function as a pump, but through interactions of the pump with heme *(21)*.

The SP fraction in the mouse mammary gland is enriched for long-term BrdU retaining LRCs as well as Sca-1 positive cells *(13)*. Despite the toxicity of the Hoechst dye, transplantation of mouse mammary SP cells at limiting dilution into cleared mammary fat pads generates epithelial ductal and alveolar structures *(13,15)*. However, enrichment in stem/progenitor activity has not been observed in the isolated SP cells, as compared to the non-SP cell population.

It is difficult to directly estimate the frequency of SP cells in the normal mammary gland, since this may be influenced by the method of isolation, the intrinsic toxicity of the Hoechst dye, and changes which may occur even during short-term primary culture of epithelial cells (see Smalley and Clarke, this issue). With these caveats, the SP population appears to represent approximately 0.5% or 1 per 200 cells. Thus, the SP population is 5- to 10-fold larger than the estimate of the frequency of stem cells determined by limiting dilution experiments, raising the need for

other molecular markers for isolating and identifying mammary stem cells.

Approximately 8% of the cells in the SP population retain BrdU, but even this subpopulation of SP cells may not be totally quiescent (G. Smith, personal communication). Thus, the SP phenotype appears to be a useful surrogate for stem-like/progenitor cells, and may be comprised primarily of a "transient amplifying" progenitor population. It is interesting to note that our recent studies have indicated that the SP population of primary mouse mammary epithelial cells (MECs) are also more resistant to clinically relevant doses of radiation than non-SP cells (W. Woodward and J.M. Rosen, unpublished observations).

The mammary gland originates from the embryonic epidermis. While mammary and epidermal stem/progenitor cell populations are probably different, it is interesting to compare and contrast these stem/progenitor cell populations. For example, it has been reported that the mouse and human epidermis also contains SP cells, and that these cells constituted a subpopulation of the $\alpha 6$ integrin-positive basal cells of the mouse epidermis that are positive for Sca-1 *(22,23)*. However, the epidermal SP cells did not express particularly high levels of $\beta 1$-integrin, another marker of epidermal stem cells. In addition, they were not identical to the label-retaining population, but were cycling cells. Furthermore, keratinocytes positive for Sca-1 were located outside the stem cell-containing bulge area of the hair follicle.

Sca-1, also designated as Ly-6a, is a GPI-anchored membrane protein and a member of the Ly-6 family. It is expressed by murine bone marrow and muscle stem cell populations, and may function in T-cell activation or cell adhesion *(24)*. Sca-1-positive mammary epithelial cells are capable of producing mammary outgrowths after transplantation into cleared murine mammary fat pads *(13)*. In this case, limiting dilution reconstitution experiments have demonstrated that a population of cells in mouse mammary gland primary cultures expressing Sca-1 was required for outgrowth. One thousand Sca-1-enriched mouse primary culture cells were able to reconstitute the mammary glands of host mice cleared of the endogenous tissue *(13)*. Moreover, transplantation of Sca-1-depleted primary culture cells resulted in poor outgrowth rates. These results established that functional mammary stem/progenitors cells can be isolated using Sca-1. Unfortunately, while other members of the Ly-6 family are conserved between mouse and human, no Ly-6a homologue has yet been identified in the human genome.

The Sca-1 phenotype appears to have been retained in the immortalized COMMA-D mammary epithelial cell line derived originally from mid-pregnant Balb/c mice *(25* and Dr. Marie-Ange Deugner, personal communication*)*. Since Sca-1 is expressed on about 20–30% of freshly prepared mouse mammary primary cells *(13)*, the Sca-1 positive cells in the mammary gland likely comprise a mixed population containing stem, progenitor and possibly some differentiated cells. In addition to transplantation studies, using a Sca-1-GFP knockin mouse, highly GFP-positive cells are located in the terminal end buds (TEBs). The brightest GFP expressing cells are found at the tips of growing ducts in what appears to be the highly proliferative cap cell layer, and weaker GFP-expressing cells are scattered along the mature ducts.

Interestingly, a subpopulation of cells in the TEBs appear to be doubly positive for keratin 6 as well as Sca-1 (S. Grimm and J.M.Rosen, unpublished observations). Keratin-6 expression is normally restricted to the body cells of TEBs during ductal morphogenesis and is rarely observed in the mature gland *(26,27)*. Keratin-6 is, however, normally thought to be a marker of hyperproliferation, especially in the skin during wound healing *(28)*. Therefore, it was somewhat surprising that keratin-6 expression was not observed in the highly proliferative mammary epithelial cells during pregnancy, but it was highly expressed in Wnt-1 induced hyperplasias *(*see below and *27)*. During embryonic mammary gland development keratin-6 expression has also been detected in the mammary anlagen (S. Grimm, personal communication). Thus, keratin 6 may also provide a marker for early mammary progenitors.

In summary, while the functional significance, if any, of BCRP1, Sca1, and K6 in mammary stem/progenitor cells remains to be established, these genes have provided useful markers for stem/progenitor cell isolation and characterization. Furthermore, as detailed in the following section, these markers have provided important insights into the etiology of a variety of different mouse models of mammary cancer with implications for understanding the heterogeneity of human breast cancer.

DO BREAST CANCERS ARISE FROM STEM CELLS?

As discussed in the Introduction, stem cells have been hypothesized to be the cells of origin of human cancers. There is strong evidence to support this hypothesis in the case of teratocarcinomas and leukemias *(29,30)*. However, the cellular origin of breast cancer has been controversial. It is still technically challenging to directly ask whether breast cancers arise from stem cells or their more differentiated progeny. However, several outcomes can be expected for cancers that do arise from stem cells: 1) their premalignant lesions may have an expanded pool of stem cells, 2) a subset of cells within the tumors may retain markers of stem cells, 3) they may be comprised of multiple cell types, due to remnant differentiation of transformed stem cells, and, 4) deregulation of genes that normally regulate stem cell proliferation and differentiation may be found in these cancers. Although cancers that arise by dedifferentiation of differentiated cells may also have these features, the evidence for dedifferentiation is scarce for most cancers that have been studied to date (reviewed in *31*).

Several signaling pathways are implicated in regulating mammary stem cells. Examples of these pathways are Wnt, Notch, Hedgehog, and TGF-β. Dysregulation of these signaling pathways in the mammary gland has been demonstrated to induce mammary tumors in genetically engineered mice *(32,33)*. Stem cell studies of other tissue types have demonstrated that Wnt signaling plays a critical role in proliferation of stem cells and maintenance of their "stemness" *(34–37)*. Therefore, we and others asked whether alteration of Wnt signaling in the mammary gland may cause an expansion of stem cells and consequently predispose them to tumors. Indeed, cells expressing keratin 6 are expanded in hyperplastic glands in both MMTV-Wnt-1 and MMTV-β-catenin transgenic mice, and cells expressing Sca-1 are also increased in mammary glands in MMTV-Wnt-1 transgenic mice *(27)*. Furthermore, SP cells are increased by 15- and 3-fold in mammary glands in MMTV-β-catenin and in MMTV-Wnt-1 transgenic mice, respectively, in comparison to littermate controls *(38)*. In addition, the potential of mammary cells from these transgenic lines to regenerate mammary glands after transplantation into cleared-fat pads is increased compared to that of the cells from littermate controls *(38* and Zhijun Du and YL, unpublished*)*, although it is not known if this is unique to mammary cells in these lines or a general feature of all oncogene-stimulated mammary cells of transgenic mice.

In addition to the expansion of putative stem cells in these transgenic mice, stem cell markers are also detected in tumors in these mice. Keratin 6 is found in mammary tumors induced by either MMTV-Wnt-1 or MMTV-β-catenin, and Sca-1 is expressed in tumor cells in MMTV-Wnt-1-induced tumors *(27)*. Consistent with observations in human cancers that only a small subset of tumor cells are tumor stem cells, only a small fraction of tumor cells in these models are keratin 6-positive, and the SP fraction of tumor cells in both Wnt and β-catenin transgenic mice is similar to that of normal mammary gland. However, Sca-1 is present in a larger fraction (60%) of tumor cells, probably reflecting its expression in both stem cells, progenitor cells, and some differentiated cells. In contrast to tumors induced by deregulated Wnt signaling, tumors induced by transgenic expression of Neu or polyoma middle T antigen (PyMT) do not express either keratin 6 or Sca-1 *(27* and see below), suggesting that different oncogenes may either target distinct mammary progenitors or promote expansion of distinct mammary cell lineages and/or differentiation programs during tumorigenesis.

In support of the possibility that Wnt signaling pathway-induced cancers arise from stem cells that retain the ability to continue to differentiate into multiple cell types, we have found that pulmonary metastasis may also contain transformed progenitor cells based on the staining for keratin 6 and on the presence of multiple cell types including myoepithelial cells (YL, unpublished observations). Collectively, these data suggest a hypothesis that activating Wnt signaling induces transformation of stem/progenitor cells, which can continue to self-renew and differentiate into multiple cell types in tumors.

Cellular heterogeneity within a tumor can be indicative of continual differentiation of transformed stem cells. We and others have found that tumors in these genetically engineered mice contain both differentiated epithelial cells and myoepithelial cells *(27,38,39)*. It should be noted that these myoepithelial cells are not normal cells recruited to the tumors because they have large pleomorphic nuclei and are disorganized. A more direct evidence that both epithelial and myoepithelial tumor cells arise from differentiation of a common progenitor comes from

the following experiment: mammary tumors arising in a cross between Wnt-1 and Pten± mice are comprised of approximately equal numbers of epithelial and myoepithelial cells; however, 70% of these tumors lose the wild type Pten allele completely when assayed by Southern blotting and by immunohistochemical staining using antibodies against Pten *(27)*. Since it is unlikely that these two cell types sustained mutations independently, the mutation probably occurred in precursors to these differentiated tumor cells.

The majority of human breast cancers do not express appreciable amounts of myoepithelial cell markers. Interestingly, a subset of human breast cancers have been identified which express myoepithelial cell markers and keratin 17, a binding partner of keratin 6. These breast cancers have also been shown to upregulate components of Wnt signaling, including c-Myc, whose transgenic expression also leads to tumors of mixed cell types including myoepithelial cells and keratin 6-positive cells *(40–43)*. This subset of human breast cancers has also been hypothesized to arise from stem/progenitor cells *(44)*.

DO BREAST CANCER ARISE FROM MORE DIFFERENTIATED CELLS?

Cancers may also arise from nonstem or differentiated cells. Cells that have lost the ability to divide may reenter the cell cycle and evolve into tumors if an oncogene can drive these cells back into cell cycle. Although their long life-span allows stem cells to accumulate multiple genetic alterations, differentiated cells may also gain a mutation that can increase their life-span or immortalize them so that they will have a chance to accumulate additional mutations and eventually evolve into cancers. Unless dedifferentiation is involved, stem cell markers and nonepithelial cells are unlikely to be present in cancers that arise from these cells.

Tumors induced by the MMTV promoter-regulated overexpression of Neu and other genes (H-Ras and PyMT) that regulate the Neu signaling pathway lack the Sca-1 and keratin 6 markers and have no myoepithelial tumor cells *(27)*, suggesting that Neu signaling might induce tumors from differentiated cells. Consistent with this possibility, mammary glands of mice carrying abnormally expressed Neu are enriched for differentiated ductal and alveolar epithelial cells, but contain few keratin 6-postive cells *(27,45)*. In addition, the tumorigenic potential of Neu is severely inhibited if Neu is activated in the early phase of mammary development or in mammary cells whose differentiation fate has been impaired *(45,46)*.

In a recent report, Wagner and colleagues *(47)* created triple transgenic mice that carry the MMTV-Neu and WAP-Cre transgenes and the ROSA-STOP-LacZ reporter construct, which expresses LacZ only after the Cre recombinase has deleted the intervening STOP sequence between the ROSA promoter and LacZ. Most of the preneoplastic and neoplastic lesions in this triple transgenic line express β-galactosidase *(47)*. Similar results are noted in a similar cross between MMTV-PyMT mice and these two reporter strains *(47)*. These observations suggest that MMTV-Neu induces expansion of and tumorigenesis from a subpopulation of mammary cells that express the differentiation marker WAP, though some of these cells also seem to have the potential to contribute to both epithelial and myoepithelial cells upon transplantation *(48)*.

WAP-positive cells are present as a minor subset in nulliparous mice, but are enriched in primiparous and multiparous animals; consequently, tumors evolved more rapidly in parous transgenic animals for Neu and PyMT *(47)*. However, the transcriptional activity of MMTV is also known to be enhanced during pregnancy; thus, it is not clear whether the increased tumor evolution is due to the increased pool of the cellular target, the enhanced expression of the transgene, or both. Nevertheless, consistent with the hypothesis that Neu and PyMT induce tumors from more differentiated mammary cells, these tumors express alveolar cell marker κ-casein *(45* and S. Huang and YL, unpublished observations, *49)*, but lack the expression of ductal cell marker NKCC1, a sodium, potassium, and chloride transporter *(47)*. In contrast, tumors arising in MMTV-Wnt-1 mice do not express κ-casein (S. Huang and YL, unpublished observations), but a fraction of the tumor cells do express NKCC1 *(47)*, supporting the hypothesis that Wnt induces tumors from stem cells and the transformed stem cells can continue to differentiate into heterogeneous cell types.

In summary, it appears that breast cancer may arise from both stem/progenitor cells and more differentiated cells. Cancers that do arise from stem cells may exhibit cellular heterogeneity; on the other hand, cancers that arise from more differentiated cells are likely to be more uniform in their cellular makeup. The transforming function of oncogenes seems to be influenced by the differentiation status

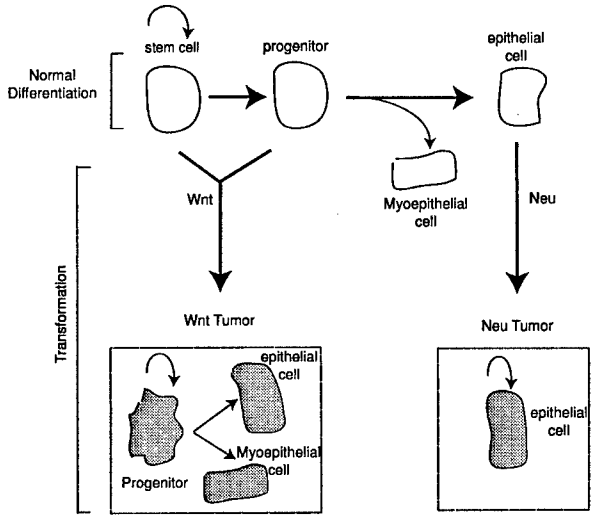

Fig. 1. Schematic model of mammary tumorigenesis induced by Wnt and Neu. Self-renewal is indicated by a half-circle with an arrowhead above the cell. Neu tumor cells are homogeneous. Wnt tumors contain transformed progenitor cells that can give rise to differentiated epithelial and myoepithelial tumor cells.

of the cell that carries the deregulated oncogenes. In another words, cells at a specific stage of differentiation may be more susceptible to transforming effects of certain oncogenic mutations, but not others (Fig. 1).

PROSPECTS

It will be important to test directly in future experiments whether the selective alterations of critical signaling pathways in stem cells will cause them to evolve into cancers comprised of both stem cells and multiple types of differentiated cells. Several potential stem cell markers are available, and more are being characterized *(50)*; thus, it may be possible to use their promoters for selective introduction of oncogenic factors to stem cells. Spatial regulation of transgene expression will be essential, since these markers are usually expressed in other organs and tissues. Furthermore, the promoter driving the oncogene should be constitutively expressed even after the stem cells have differentiated into different cell types, including myoepithelial cells. Thus, the promoter driving the oncogene should be different from the promoter used for targeting the oncogene selectively to stem cells. While tissue transplantation and rtTA or Cre-mediated conditional expression *(45, 51)* can satisfy some of these technical challenges; the TVA (tumor virus A) technology *(52)* offers an attractive alternative for flexile targeting of oncogenic events selectively to stem cells and maintaining the expression of oncogenes in any cell type to which the stem cell may differentiate.

The TVA gene transfer system is based on the use of a sub-group A avian leukosis virus vector (RCAS) to carry exogenous genes to specific somatic mouse cells that are made susceptible to infection by the expression of TVA, the receptor for RCAS *(52)*. Since mammalian cells lack this receptor, they are not normally susceptible to infection by RCAS; however, ectopic expression of TVA (e.g. by the Sca-1 promoter) transforms an otherwise resistant cell type to a susceptible one. Thus, RCAS vectors may be used to infect and deliver oncogenes at any time of mammary ductal development. Since RCAS produces only the exogenous gene product, but not viral structural proteins, this virus does not spread or induce immune rejection. Importantly, exogenous genes (such as oncogenes) cloned into this vector are

expressed from the constitutively active viral LTR and independent of the promoter controlling TVA, which directs the virus to specific cells (such as stem cells).

Thus, an oncogene delivered by this method continues to be expressed even after infected cells have differentiated. However, since RCAS requires dividing TVA^+ cells for infection, a modified HIV vector (HIV/ALV), formed by pseudotyping the HIV virus with the envelope gene of RCAS and capable of infecting all TVA^+ cells regardless of the cell proliferation status *(53)*, may be necessary to infect stem cells since they should, by definition, be relatively quiescent. The use of this technology may provide a more rapid approach to determine the effects of other stem cell regulators on inducing cancer. In addition, with this method, it may also be possible to ask whether stem cell regulators (such as Wnt or Notch) can induce dedifferentiation of differentiated breast epithelial cells if these factors are selectively transferred to differentiated cells using transgenic lines expressing TVA from a promoters of a differentiation marker (such as WAP). Such an approach would overcome the potential concern associated with conventional transgenic techniques that the expression of the test gene is shut off after the cell has dedifferentiated and stopped expressing the transgenic promoter.

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